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Regulation of the *Escherichia coli* DNA topoisomerase I gene by DNA supercoiling

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ABSTRACT

The transcriptional control region of THE E. coli DNA topoisomerase I (topA) gene has been fused to the galactokinase (galK) gene coding region in a recombinant plasmid. In vivo synthesis of the galactokinase produced from such a plasmid has been measured and found to be reduced when mutations in the genes coding for DNA gyrase subunits are introduced into the cell or when gyrase inhibitors are present. In vitro transcription-translation of the galactokinase gene product confirms that a supercoiled DNA template is required for efficient transcription from the topA gene promoter. These results indicate that the amount of DNA topoisomerase I activity in E. coli is regulated by the extent of DNA supercoiling and can contribute to the overall modulation of DNA superhelicity and the expression of other genes.

INTRODUCTION

Bacterial DNA is normally maintained in a negatively supercoiled state. It is generally believed that this normal level of supercoiling is regulated by the balance between the supercoiling activity of DNA gyrase and the relaxing activity of DNA topoisomerase I[1-3]. Transcription from various bacterial promoters is known to be modulated by DNA superhelicity in different ways[4,5]. The rates of synthesis of DNA gyrase subunit A and subunit B have been shown to be highest when the DNA template is relaxed [6]. Since DNA gyrase introduces negative supercoils into DNA, a homeostatic control mechanism for DNA supercoiling has been proposed [6]. In this paper, the effect of DNA supercoiling on the efficiency of transcription from the promoter for E. coli DNA topoisomerase I (topA) gene is being studied. The 5' control region of the topA gene is fused to the galactokinase (galK) gene. The production of galactokinase is then under the control of the topA promoter. Similar

galactokinase fusion systems have been used previously to study transcriptional control of other genes [7-9]. In this paper we assay the amount of galactokinase produced *in vivo* from such a recombinant plasmid and show that it is reduced when DNA supercoiling by gyrase is inhibited by the specific inhibitors novobiocin and nalixidic acid or mutations in the gyrase genes. We also use the recombinant plasmid as a substrate in an *in vitro* bacterial cell free extract directed transcription-translation system. Supercoiling of the DNA substrate is found to be required for efficient transcription from the *topA* promoter. This thus provides a means of regulation of the amount of DNA topoisomerase I produced in *E. coli*.

MATERIALS AND METHODS

Plasmid Construction

Restriction and other DNA modifying enzymes were obtained from New England Bio Labs unless otherwise stated. The plasmid pID35 was kindly provided by Dr. J. C. Wang of Harvard University. It contains the *Pst*I to *Eco*R1 fragment of the *cysB-topA-trp* region of *E. coli* [10] cloned into the *Hind*III site of pBR322. After *Hpa*II digestion of this plasmid, a 513 bp fragment was obtained. From DNA sequence analysis of the *topA* gene (Y.-C. Tse, J. C. Wang, manuscript in preparation), this fragment is known to include the sequence coding for the first ten amino acids of DNA topoisomerase I, as well as 483 nucleotides immediately upstream of the ATG coding for the starting f-met. The *Hpa*II digestion mixture of 10 µg of pID35 was electrophoresed in a 4% polyacrylamide gel with 90 mM Tris-borate pH 8.3, 2 mM EDTA buffer. After ethidium bromide staining, the 513 bp fragment was excised over a long wavelength UV source and diced into 1 mm squares avoiding crushing of the gel. The gel pieces were incubated overnight at 37°C in 0.8 ml of 0.5 M NH_4OAc, 1 mM Na_3EDTA to elute the DNA. The DNA was then ethanol precipitated twice and rinsed with 70% ethanol.

The *Hpa*II 513 bp fragment was next cloned into the pUC8 plasmid (Fig. 1a) so that it would be spanned by the appropriate restriction sites. The ends of the fragment were filled in with the Klenow fragment of *E. coli* DNA polymerase I [11]. pUC8 DNA (obtained from P-L Biochemicals) was digested with *Hinc*II, phenol

extracted and ethanol precipitated. 0.7 pmole of the vector was mixed with 2.1 pmole of the HpaII fragment and ligated with T4 ligase (2.5 Weiss units from Collaborative Research at 16°C overnight). To eliminate product from self-ligation of pUC8 vector, the ligation mixture was incubated with 10 units of SalI for 60 minutes before being used to transform competent JM103 E. coli cells. Two types of recombinant plasmids were obtained, each containing the insert with the topA gene 5' control sequence in a different orientation (Fig. 1a) and named pUCIB1 and pUCID1.

Fusion to the galK gene was accomplished using pDR540, a derivative of pKO-1[7], purchased from P-L Biochemicals. Digestion with BamH1 and HindIII removed the promoter upstream of the galK gene and galactokinase was not expressed when the digested vector was ligated by itself after the ends were filled in. This vector fragment and the fragment from digestion of plasmid pUCIB1 with BamH1 and HindIII that contained the topA gene 5' upstream sequence were both isolated by excision from a 1% low melting point agarose (purchased from BRL) gel electrophoresed in buffer of 50 mM Tris-HCl pH 8, 20 mM NaOAc, 1 mM Na_3EDTA buffer. The two gel slices were combined in a 1.5 ml eppendorf centrifuge tube, melted at 65°C and diluted with three volumes of 10 mM Tris HCl pH 8, 0.1 mM Na_3EDTA buffer. One-tenth final volume of 10X ligase buffer (200 mM Tris HCl, pH 7.5, 70 mM $MgCl_2$, 100 mM DTT, 1 mg/ml gelatin) was added. The ATP concentration was adjusted to 1 mM. 12.5 units of T4 ligase was added. Ligation was carried out at 16°C for 18 hours (scheme shown in Figure 1b). The ligation mixture was used to transform E. coli (C600 galK$^-$) cells. Transformants with the resulting pBDR1 plasmid had a galK$^+$ phenotype and appeared as red colonies on MacConkey agar plate with 1% galactose [7]. The structure of pBDR1 was confirmed by restriction enzyme analysis.

Bacterial Strains

E. coli strain C600 was obtained from P-L Biochemicals. It was used for growth of the topA-galactokinase fusion plasmid DNA and as a parent strain for construction of other strains. P1 lysates were prepared from strains with Tn10 inserts linked closely to either gyrA or gyrB with or without a mutation. The P1 lysates were then used as donors in transduction of C600. The

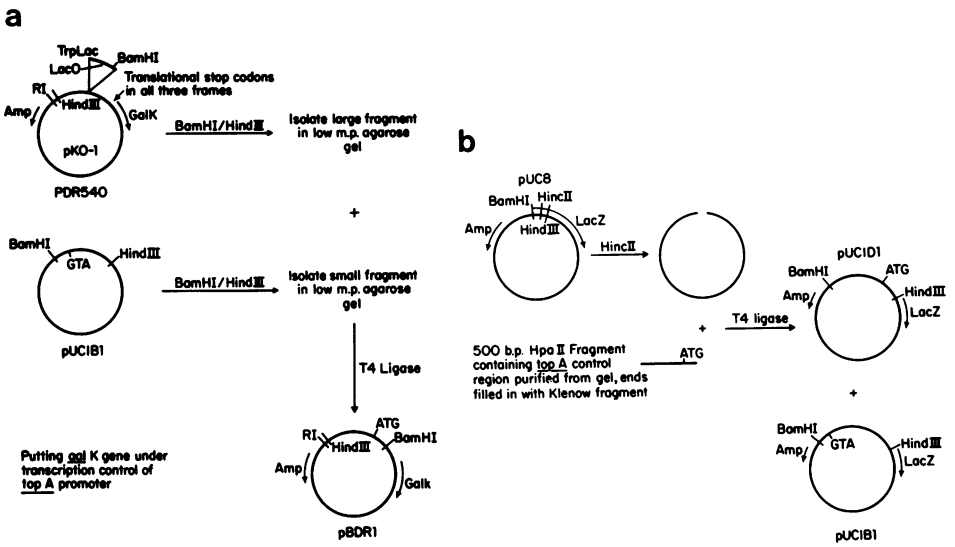


Figure 1
(a) Scheme of subcloning of DNA fragment containing 5' upstream control sequence of topA gene
(b) Scheme of construction of pBDR1 - a plasmid with galactokinase gene under the transcriptional control of topA gene

relevant genotypes and sources of the various strains used are listed in Table 1. E. coli cells with mutations in gyrA or gyrB genes have been shown to express a Bgl$^+$ phenotype[2]. Following selection of tetracycline-resistant (Tn10-containing) recombinants, transductants were thus screened for presence of gyrA or gyrB mutations by their color on MacConkey agar plates with 0.1% bromothymol blue and 0.5% salicin[2]. Fermentors (Bgl$^+$) appear as orange colonies. Nonfermentors (Bgl$^-$) appear as whitish-gray colonies and have gyrA$^+$ gyrB$^+$ genotype.

Galactokinase Assays

Galactokinase assays were performed as described previously[7]. Cells containing pBDR1 (200 ml volume) were grown to exponential phase in M-56 medium with 0.2% fructose as carbon source. When gyrase inhibitors were used, they were added to cells grown to an OD_{650} of 0.4. Cell growth was then continued until the OD_{650} reached 0.7 - 1.0. A 1.0 ml sample of cells was used for preparation of the cell lysate for assay[7]. D-[1-^{14}C]-galactose (30 - 50 Ci/mmol) was obtained from NEN. Galactokinase

TABLE 1 BACTERIAL STRAINS USED IN THIS WORK

Strain	Relevant Genotype	Source or Derivation
C600	*galK*$^-$	PL Biochemicals
K0635	*tna7*::Tn*10*[a]	(2)
SD104-20	*gyrB800 tna7*::Tn*10*	(2)
CSD01	*galK*$^-$ *tna7*::Tn*10*	P1(K0635)xC600→Tet^r(Bgl$^-$)
CSD5	*galK*$^-$ *gyrB800 tna7*::Tn*10*	P1(SD104-20)xC600→Tet^r(Bgl$^+$)
RM101	*zei*::Tn*10*[b]	R. Menzel
KV4752	*gyrA750*	(3)
KVR1	*gyrA750 zei*::Tn*10*	P1(RM101)xKV4752→Tet^r
CKR01	*galK*$^-$ *zei*::Tn*10*	P1(KVR1)xC600→Tet^r(Bgl$^-$)
CKR5	*galK*$^-$ *gyrA750 zei*::Tn*10*	P1(KVR1)xC600→Tet^r(Bgl$^+$)

[a] *tna* and *gyrB* are cotransducible
[b] *zei* and *gyrA* are cotransducible
P1(X)xY Z(Q) indicates that strain X was the transduction donor and strain Y the recipient in a bacteriophage P1 mediated genetic cross. Z was the selected marker and Q the unselected cotransducing marker.

units are expressed as nanomoles of galactose phosphorylated per minute per ml of cells at OD_{650} of 1.0.

β-Lactamase Assay

0.1 ml of cell lysate prepared in the same way as for galactokinase assay was mixed with 0.9 ml of 50 mM potassium phosphate pH 7.4 in a cuvette at room temperature and placed in a Perkin-Elmer Lambda 5 UV/Vis Spectrophotometer. Absorbance at 486 nm was adjusted to zero. 0.1 ml of nitrocefin[15] (0.1 mM in 10 mM sodium phosphate pH 7.5, 5% dimethyl sulfoxide) was then mixed in. Absorbance readings at 486 nm were taken every 15 seconds for 2.5 minutes and plotted to obtain the initial velocity of the reaction.

In Vitro Transcription-Translation

An *E. coli* cell free transcription-translation kit was purchased from Amersham. Protein synthesis using pBDR1 plasmid DNA as template was carried out according to the protocol provided with the kit. L-[^{35}S]methionine (translation grade, 800 Ci/mmole) was obtained from NEN. 0.5 - 2.5 µg of plasmid DNA was used in each assay in a final volume of 35 µl. Gyrase inhibitors, when included in the experiment, were added to the plasmid DNA before the S30 cell free extract was added. SDS gel electrophoresis analysis was carried out according to Laemmli[12]. 5 µl of the final sample was added to 5 µl of 2 x loading buffer and heated for 5 minutes at 100°C, then loaded onto a 10 or 15%

SDS-polyacrylamide gel with a 5% stacking gel. After electrophoresis, gels were fixed in 7% acetic acid for 30 min., dried with a Bio-Rad gel dryer, and then autoradiographed. Autoradiograms of different exposure times were scanned with a Helena densitometer. To check the superhelical state of the DNA template at the end of the reaction, 15 µl of the sample was added to 5 µl of 50 mM EDTA, 50% glycerol, 0.1 mg/ml bromophenol blue and electrophoresed in a 0.8% agarose gel in TBE (90 mM Tris-borate pH 8.3, 2 mM EDTA) buffer. The gel was then stained in 0.5 mg/l ethidium bromide and visualized with UV light.

The supercoiled plasmid DNA template was purified from a cesium chloride-ethidium bromide gradient as described [10] and contained < 5% contaminating open circles. Relaxed plasmid DNA template was prepared by incubation of the supercoiled molecules with calf thymus DNA topoisomerase I (purchased from BRL) followed by phenol extraction and ethanol precipitation.

Gyrase Inhibitors

Novobiocin and coumermycin were purchased from Sigma. Nalixidic acid was obtained from Boehringer Mannheim.

RESULTS

In Vivo Expression of Galactokinase Under Transcriptional Control of the topA Gene Promoter is Reduced When Gyrase Activity is Low

Table 2 summarizes the results of the measurements of *in vivo* expression of the *galK* gene on pBDR1. Galactokinase activity is reduced when DNA gryase is inhibited by either nalixidic acid or novobiocin. Comparing galactokinase activity expressed from pBDR1 in isogenic strains with or without gyrase mutations, galactokinase activity is lower in cells with the gyrase mutations. The gyrase mutations have been shown previously to cause a lower level of DNA supercoiling[3]. These results suggest that transcription from the *topA* promoter is reduced when DNA supercoiling is inhibited. Plasmid pBDR1 DNA has also been isolated from the *E. coli* cells used in these measurements by the rapid boiling method [13] as well as the SDS/Ficoll lysis method used previously for copy-number determination [14]. Electrophoresis of the plasmid DNA indicates that the copy number of the plasmid does not vary in the *E. coli* cells used for galactokinase activity measurement. The effects

TABLE 2
Galactokinase activity expressed *in vivo* under the transcriptional control of *topA* gene is reduced by the treatment with DNA gyrase inhibitors or mutations in DNA gyrase genes.

E. coli Cells Containing pBDR1	Galactokinase Units Measured
C600 without pBDR1	2
C600	96
C600 treated with 50 µg/ml nalixidic acid	51
C600 treated with 1 mg/ml novobiocin	24
CKR01 (*gyrA*$^+$)	116
CKR5 (*gyrA750*)	39
CSD01 (*gyrB*$^+$)	113
CSD5 (*gyrB800*)	57

of the inhibitors and mutations on the superhelicity of the pBDR1 plasmid DNA have been confirmed by electrophoresis of the purified DNA in agarose gels containing the intercalator chloroquine[16]. β-lactamase activity in the cell lysates has been measured. It is not affected by the gyrase inhibitors as the galactokinase activity is so protein synthesis in general is not inhibited. Thus reduction in *topA* promoter-specific transcription is probably responsible for the lowering of galactokinase activity.

In Vitro Transcription-Translation of the Galactokinase Gene Under the Control of the topA Promoter is Dependent on the Supercoiling of the DNA Template

Supercoiled pBDR1 plasmid DNA functions as an efficient template in an *E. coli* extract directed *in vitro* transcription-translation reaction (Figure 2, lane c). The *topA* promoter appears intermediate in strength in this system when compared to the *Trp-Lac* and *amp* promoters. pBDR1 DNA linearized at the *EcoR1* site is a much less efficient template (lane d). The *amp* promoter directed transcription is down in this substrate because of the proximity of the *EcoR1* cut to the -35 region of the promoter. Relaxed pBDR1 DNA is almost as efficient as supercoiled pBDR1 when added to the standard reaction mixture (lane e). However, relaxed pBDR1 DNA in the presence of 0.1 mg/ml of novobiocin again gave a greatly reduced amount of galactokinase (lane f). Transcription-translation in general is not inhibited by this concentration of novobiocin since β-lactamase synthesis from the same plasmid is not affected.

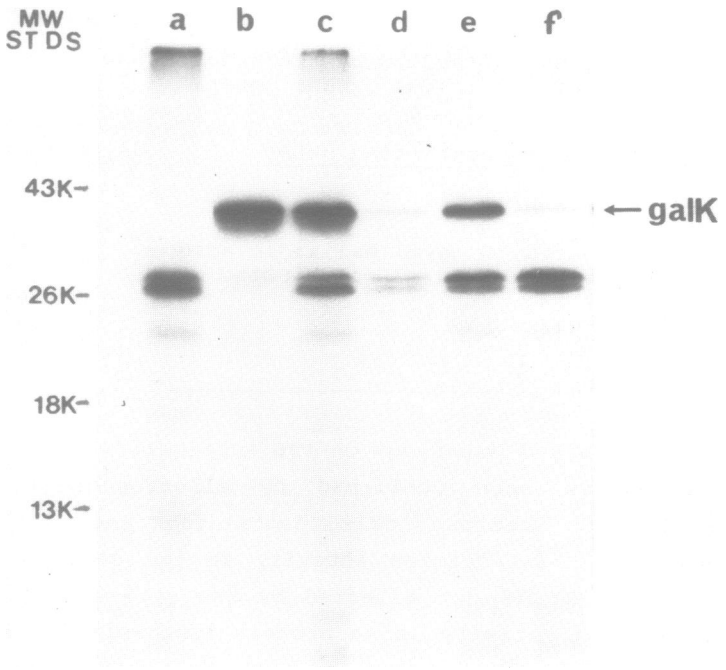


Figure 2

Efficiency of In vitro synthesis of Galactokinase from pBDR1 is highly dependent on the conformation of DNA template
Autoradiogram of a 15% SDS polyacrylamide gel following electrophoresis of the products of in vitro transcription-translation reactions
The DNA templates were 2.5 µg of:

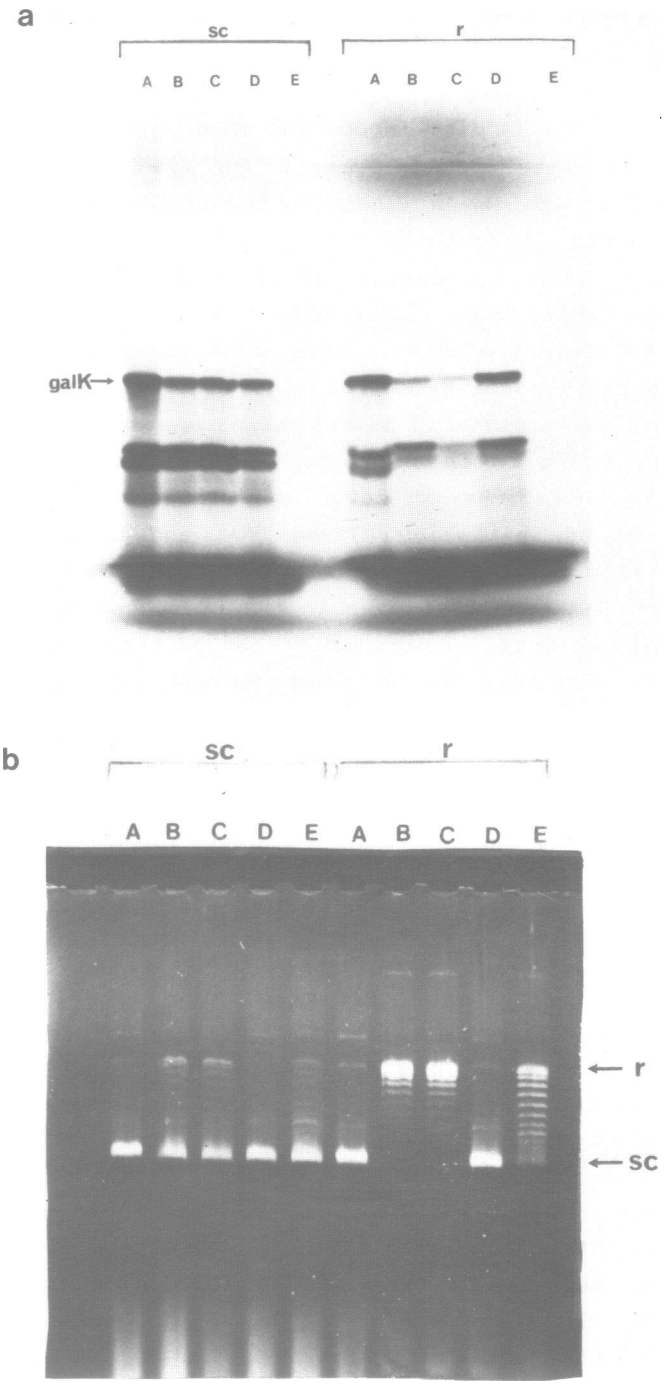
a) supercoiled pAT153 plasmid (supplied with the kit), coding for β-lactamase only
b) supercoiled pDR540
c) supercoiled pBDR1
d) pBDR1 linearized by EcoR1
e) relaxed pBDR1
f) relaxed pBDR1 with 0.1 mg/ml novobiocin

Examination of the plasmid DNA template by agarose gel electrophoresis shows that the initially relaxed pBDR1 DNA in lane e has been converted to the supercoiled form at the end of the in vitro transcription-translation reaction. This supercoiling is inhibited by novobiocin and the DNA template remains relaxed in its presence. This again suggests that in vitro transcription from the topA promoter is dependent on the supercoiling of the DNA template.

Figure 3 shows the effects of various concentrations of novobiocin and nalixidic acid on *in vitro* transcription-translation of the galactokinase from pBDR1 and the DNA topology at the end of the 30 min. incubation when initially either supercoiled or relaxed pBDR1 plasmid DNA is used as template. Nalixidic acid at 1 mg/ml inhibits the transcription-translation reactions (lane E) probably due to effects unrelated to gyrase inhibition. Lower concentrations of nalixidic acid (lane D and other experiments) have little effect on the supercoiling of the relaxed DNA template under these conditions and galactokinase production is thus not affected significantly. Therefore, unlike novobiocin, nalixidic acid is not useful as a specific inhibitor of DNA supercoiling in this *in vitro* system of gene expression. Various concentrations of novobiocin (lanes B, C) inhibit the supercoiling of initially relaxed DNA, and hence galactokinase synthesis from the *topA* promoter (10-20 fold). However, initially supercoiled DNA template, even in the presence of novobiocin, is relaxed only slightly at the end of the 30 min. incubation and so the effect of novobiocin on galactokinase synthesis is small (1.5 fold). These results indicate that the transcription from the *topA* promoter is regulated by the supercoiling of the DNA template rather than DNA gyrase itself directly. Coumermycin (0.3 - 3 µg/ml), another specific inhibitor of the gyrase B subunit, gave similar results to novobiocin.

DISCUSSION

The *in vivo* and *in vitro* results presented here can be explained by the proposal that efficient transcription from the *topA* promoter requires negative supercoiling of the DNA template. A similar conclusion has been obtained by R. Menzel and M. Gellert (personal communication) using immunoprecipitation to monitor *topA* expression in *E. coli*. They have previously reported that synthesis of DNA gyrase is highest when the DNA template is relaxed and proposed a homeostatic mechansism for control of DNA supercoiling[6]. The depression of transcription of DNA topoisomerase I when DNA is relaxed presumably results in a decreased level of topoisomerase I activity and decreased capacity of the cell to relax supercoiled DNA. It is not clear


a
sc
r
A B C D E
A B C D E
galK
b
sc
r
A B C D E A B C D E
r
sc

how the effects of DNA gyrase and DNA topoisomerase I interplay against each other in different stages of cell growth because DNA superhelicity may vary in coordination with the cell cycle. The most recently discovered type I topoisomerase in E. coli, DNA topoisomerase III[17], and other DNA binding protein in E. coli may also play a role in the regulation of global or local DNA superhelical density.

The molecular mechanism by which DNA superhelicity controls transcription from the topA promoter is not known. The negatively supercoiled state of the template is required for direct interaction between the template and RNA polymerase, or for the effective binding of a positive stimulator, or for the dissociation of a repressor. The fact that a supercoiled DNA template in the in vitro transcription-translation system is relatively insensitive to gyrase inhibitors argues against DNA gyrase itself as a stimulator. It has been reported that the introduction of a multicopy plasmid containing the regulatory region of the topA gene but not the coding region does not have much effect on the level of DNA topoisomerase I produced from the genomic copy of topA [4]. Therefore, the cellular level of any stimulator or repressor, if present, has to be fairly high.

E. coli RNA polymerase is known to unwind DNA strands (18-24) upon binding to a promoter and therefore is predicted thermodynamically to bind more strongly to negatively supercoiled DNA. The overall level of transcription in E. coli is decreased

Figure 3

Effect of various concentrations of novobiocin and nalixidic acid on in vitro transcription-translation of galactokinase gene under the control of the topA promoter

0.5 µg of either supercoiled (sc) or relaxed (r) pBDR1 DNA was used as the template in a standard in vitro transcription-translation reaction with

Lanes (A) control, no drug
(B) 0.03 mg/ml novobiocin
(C) 0.3 mg/ml novobiocin
(D) 0.1 mg/ml nalixidic acid
(E) 1 mg/ml nalixidic acid

a) Autoradiogram of a 10% SDS polyacrylamide gel analyzing the ^{35}S-labeled polypeptides synthesized

b) Photograph of ethidium bromide stained agarose gel analyzing the topological state of the DNA template at the end of the in vitro transcription-translation reaction.

by gyrase inhibitors so transcription of most promoters is probably enhanced by negative supercoiling[5]. However, several promoters including gyrA, gyrB, trp, bgl, thr, and gal have been shown to have rates of transcription that are either unaffected or enhanced by decrease in negative superhelicity of the template[4]. Each promoter has a different rate-limiting step depending on its sequence, intracellular RNA polymerase concentration, and binding of regulatory elements. The effect of supercoiling on transcription is therefore a complex one. Work is currently in progress to determine if the -35 and -10 sequences of the topA promoter are solely responsible for the dependence of transcription on a negatively supercoiled DNA template.

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